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A photoactivatable synthetic transit peptide labels 30 kDa and 52 kDa polypeptides of the chloroplast inner envelope membrane

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A 24-residue transit peptide based on the sequence of a precursor of the small subunit of wheat ribulose-1,5-bisphosphate carboxylase (Rubisco) was synthesized. The transit peptide was converted into a radioactive azido derivatised analogue. Photoactivation of the radiolabelled transit peptide analogue with isolated inner and outer membranes of the chloroplast envelope intensely labelled two proteins of 30 kDa and 52 kDa. In the outer membrane only the 52 kDa polypeptide was labelled. These findings are consistent with a recent report on the identification of the 30 kDa receptor protein for protein import in the chloroplast envelope contact zones [(1988) Nature 331, 232–237].

Transit peptide; Chloroplast; Import receptor

1. INTRODUCTION

A vast majority of higher plant chloroplast proteins are encoded by nuclear genes and are synthesized on cytoplasmic ribosomes. These proteins are post-translationally imported into the chloroplast by an energy-dependent process [1]. The chloroplast proteins after synthesis on the cytoplasmic ribosomes carry transit peptide extensions which are instrumental in specific recognition of the chloroplast surface and subsequent translocation and segregation of proteins to their functional compartments within the chloroplast [2]. That the chloroplast envelope surface carries specific receptors which recognise transit peptide was first demonstrated in experiments where the precursor protein import capability of the isolated intact chloroplast was abolished following mild proteolysis [3]. Isolated chloroplast envelope fractions also demonstrate selective binding of the precursor proteins carrying transit peptides [4,5]. Recently Pain and co-workers [6] using an idiotypic antibody raised against a synthetic transit peptide confirmed the presence and identity of a 30 kDa import receptor protein localised in the chloroplast envelope. Here we report cross-linking to a similar 30 kDa polypeptide in the inner membrane of the chloroplast envelope by a synthesized 24-residue photoactivatable transit peptide. Our results based on a different approach are in agreement with those reported by Pain et al. [6]. The 52 kDa large subunit of Rubisco is also a major target protein with which the transit peptide interacts.

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Abbreviations: ASTP, Met^1-(4-azidosalicyl),Lys^{20}-(4-azidosalicyl) transit peptide; IEF, isoelectric focussing; PAGE, polyacrylamide gel electrophoresis; Rubisco, ribulose-1,5-bisphosphate carboxylase

2. MATERIALS AND METHODS

The transit peptide of the small subunit of wheat (*Triticum aestivum*) Rubisco comprising the first 24 residues (fig.1a) was synthesized on a polystyrene support, using temporary protection of amino groups by the fluorenyl methoxycarbonyl method [7], employing a Vega Computer Automated Peptide Synthesiser. After deprotection, the peptide was partially purified by gel filtration on Sephadex G-15 in 10 mM acetic acid. It was

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further purified by HPLC on a Brownlee Aquapore C-8 reverse-phase column applying a linear gradient of acetonitrile ranging from 0 to 70% and characterised by analyses of its amino acid composition; Thr 2.98 (3), Ser 2.82 (3), Glx 1.15 (1), Pro 2.1 (2), Gly 2.15 (2), Ala 6.02 (6), Val 1.94 (2), Met 1.95 (2), Leu 1.06 (1), Phe 0.83 (1) and Lys 1.1 (1), and determination of its amino- and carboxy-terminial residues.

A photoactivatable analogue of the transit peptide was prepared by mild esterification of 2 mg of the peptide by incubation with 135 μg of *N*-hydroxysuccinimidyl-4-azidosalicylic acid (Pierce and Wariner, England) in a final volume of 200 μl containing 0.1 M PO_4 buffer, pH 7.5, at 20°C in the dark for 14 h [8]. Radioiodination was carried out in the same reaction mixture by adding 1 mCi of carrier-free ^{125}I (Amersham International) in the presence of chloramine T [9]. The radiolabelled product was desalted on Biogel P-2.

The chloroplast envelope membranes were prepared from intact chloroplasts isolated from 10-day old pea (*Pisum sativum* var. Feltham first) leaves essentially by the procedure described by Dounce and Joyard [10]. The inner and outer membranes were separated by centrifugation on a discontinuous sucrose density gradient by the method of Cline et al. [11].

3. RESULTS AND DISCUSSION

Fig.1b shows UV spectra of *N*-hydroxysuccinimidyl 4-azidosalicylic acid before and after UV irradiation. The photoactivatable reagent displays an absorption maximum at around 353 nm which decays very rapidly upon illumination with UV light at wavelength greater than 300 nm. The transit peptide after coupling with the photoactivatable reagent showed a distinct spectrum from the uncoupled probe with an absorption maximum at 320 nm which also decayed rapidly after 1 s of exposure to UV light. The stoichiometry of the reaction between *N*-hydroxysuccinimidyl 4-azidosalicylic acid and the synthetic transit peptide was deduced to be 2:1 using the published extinction coefficient of $2 \times 10^4\ M^{-1} \cdot cm$ [12]. The radioiodinated Met^1-(4-azidosalicyl),Lys^{20}-(4-azidosalicyl) transit peptide (^{125}I-ASTP) was assessed to be labelled to a specific activity of 1.5×10^8 cmp/μmol peptide.

^{125}I-ASTP was incubated with the inner and outer membranes of chloroplast envelopes and photoactivated as described in the legend to fig.2. The radiolabelled membranes were concentrated by precipitation with trichloroacetic acid and subjected to electrophoretic analysis. The membrane proteins which were cross-linked with the transit

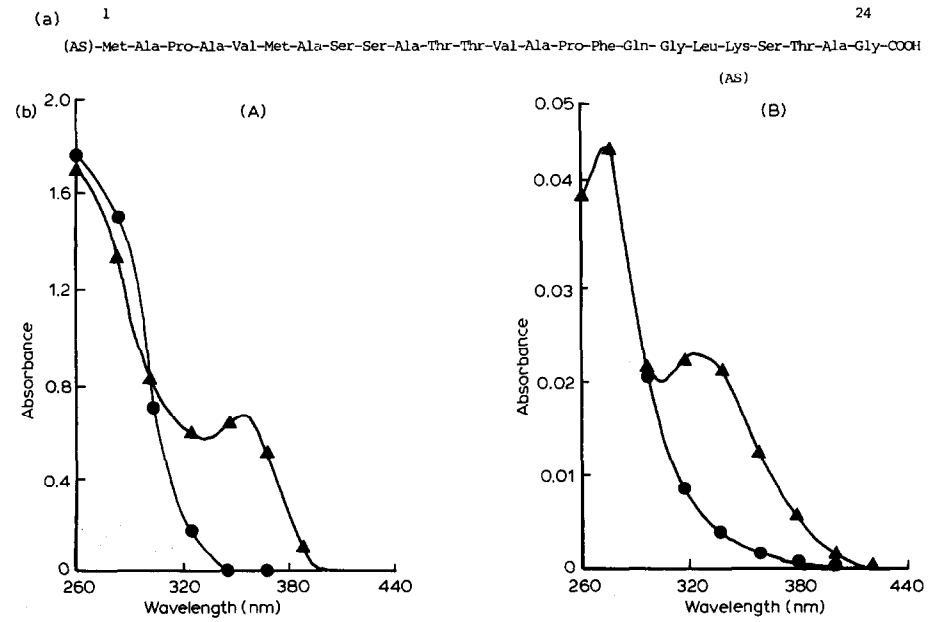

Fig.1. (a) Amino acid sequence of the transit peptide of the small subunit of wheat Rubisco. The sequence is based on the nucleotide sequence of a cDNA to rbc S described by Broglie et al. [16]. The probable azidosalicylated residues are indicated by AS. (b) Spectra of (A) *N*-hydroxysuccinimidyl-4-azidosalicylic acid (200 μM) and (B) ASTP (1.25 μM) before (▲—▲) and after (●—●) photoactivation. The reagents were prepared in the dark in 0.1 M phosphate buffer, pH 7.5, at the concentrations stated above and activated for 1 s as described in the legend to fig.2.

peptide analogue were visualised by autoradiography of the electrophoretogram. Fig.2 shows results of such an experiment. In the inner membrane the major radiolabelled adduct is the 30 kDa polypeptide but other radiolabelled proteins are also visible, including a 52 kDa component. A comparison of the radiolabelled bands with the silver-stained protein profile indicates that the transit peptide interacts with a small number of the polypeptides in the membrane. In contrast to the inner membranes, photolysis of the ^{125}I-ASTP with the outer membranes results in the labelling of only one protein of 52 kDa. Since no other components are labelled this lends further support to the suggestion that the transit peptide interacts with definable targets in the membrane rather than random interactions with any component.

Blobel and co-workers [6] demonstrated significantly greater immunoprecipitation of the 52 kDa large subunit of Rubisco with anti-idiotypic antibodies raised against the 30-residue transit peptide of pea small subunit of Rubisco compared with the 30 kDa polypeptide for protein import. In contrast, our photolabelling technique, employing the 24-residue transit peptide of wheat small subunit of Rubisco, labels the 30 kDa polypeptide more intensely than the 52 kDa subunit. The presence of significant quantities of large subunit of Rubisco in both the inner and outer membranes of the chloroplast envelope has been observed in numerous previous studies [4,13], and is also detectable in the silver-stained gels shown in figs 2 and 3. The precise reasons for the interaction of the transit peptide with the chloroplast-encoded large subunit remains elusive but, as has been suggested [6], the import specifying sequence may contain critical information to guide the incoming precursor into assembly with the large subunit to form the functional holoenzyme. The major labelled product linked to ^{125}I-ASTP in the inner membrane is slightly larger than the previously reported 30 kDa polypeptide import receptor, but the addition of the 3 kDa transit peptide would be expected to lead to an increase in size.

The 30 kDa polypeptide detected by silver staining appears to be the major component of the inner membrane and is clearly absent in the outer membrane (see fig.2). It is known that the major 30 kDa polypeptide in the inner membrane is the

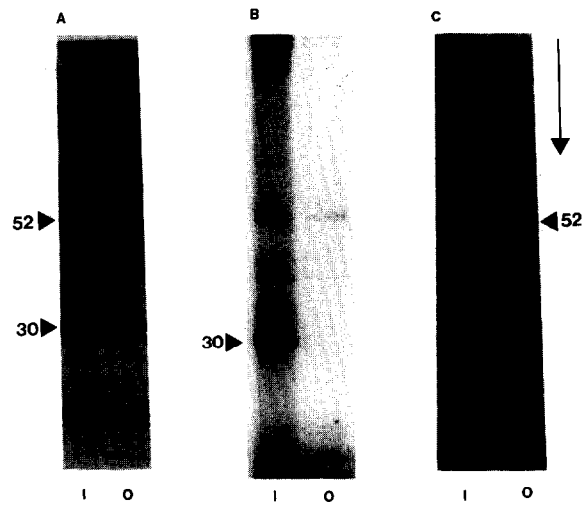

Fig.2. Labelling of chloroplast envelope membrane polypeptides by ^{125}I-ASTP. Isolated inner and outer membranes of chloroplast envelope membranes, each 100 μg, suspended in final volume of 300 μl of 0.33 M sorbitol, 10 mM Tricine/NaOH, pH 7.5, were incubated with 150 nmol ^{125}I-ASTP in nitrogen-flushed sealed pyrex tube for 2.5 min in dark after which the mixture was irradiated from a distance of 9 cm with light emitted in a wavelength greater than 300 nm from a multiband UV light torch, model SL-58 50 Hz (Ultraviolet Products, San Gabriel, CA, USA) for 15 min at 20°C. The proteins were concentrated by precipitation with 10% (w/v) trichloroacetic acid. After solubilisation in SDS sample buffer containing 5% (w/v) SDS, 20% (w/v) glycerol, 50 mM dithiothreitol, 0.005% (w/v) bromophenol blue, 25 mM Tris-HCl, pH 7.0, the samples were boiled for 1 min and then separated by electrophoresis in a gradient polyacrylamide gel ranging from 10 to 16% (w/v) in the presence of SDS: (A) shows polypeptides visualised by silver staining [17]; (B) and (C) are autoradiograms exposed for 5 and 10 days, respectively, showing polypeptides modified by ^{125}I-ASTP. I, inner membranes; O, outer membranes.

phosphate translocator which is the key exchanger of inorganic phosphate in the cytosol and phosphorylated 3-carbon sugars photosynthetically synthesized in the chloroplast [14,15]. The analysis by high resolution two-dimensional IEF/PAGE of inner membranes shows that the major 30 kDa polypeptide appears to be a singular species (fig.3) rendering it extremely likely that the species labelled by ^{125}I-ASTP is the phosphate translocator. It is possible, therefore, that the singular major 30 kDa polypeptide of the inner membrane of chloroplast envelope serves a dual function in the transport of metabolites and import of precursor proteins into the chloroplast.

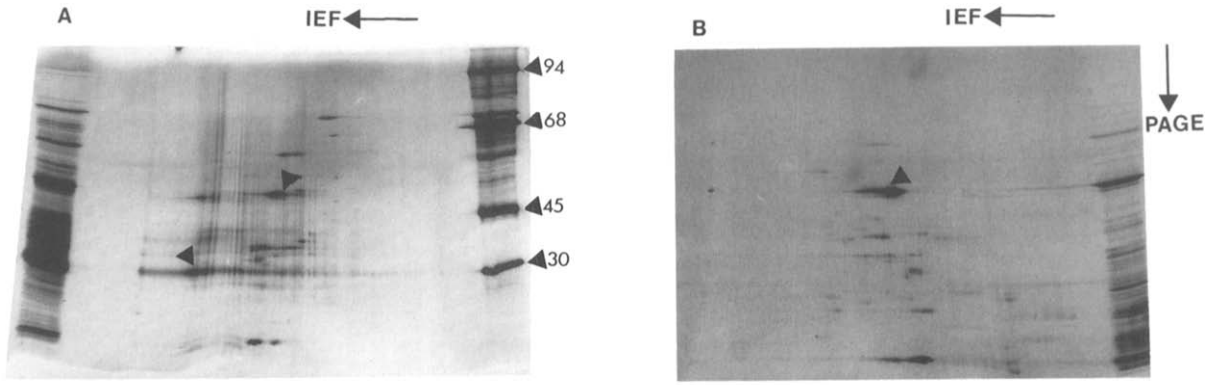

Fig.3. Two-dimensional IEF/SDS polyacrylamide gel electrophoresis of (A) inner and (B) outer membranes of pea chloroplast envelope. Separation was carried out as described in [18]. Approx. 100 μg protein was loaded and the resolved proteins were detected by silver staining. The positions of large subunit of Rubisco and the 30 kDa polypeptide are indicated. The left-hand track of (A) shows inner membrane polypeptides resolved in one-dimensional SDS-PAGE whereas the right-hand track shows marker proteins. The right-hand track of (B) shows outer membrane polypeptides separated in one-dimensional SDS-PAGE.

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